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(54) Title: A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS

(57) Abstract

This application describes a high throughtput assay for screening for compounds which are capable of binding to a fusion protein which consists of a target protein and an FK506-binding protein.

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TITLE OF THE INVENTION

A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS

BACKGROUND OF THE INVENTION

Src homology 2 (SH2) domains are a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. They have routinely been expressed in *E. coli* as fusion proteins with glutathione-S-transferase (GST). This usually provides high level expression and straightforward affinity purification on glutathione-Sepharose. Ligand binding is then assayed by incubating the GST/SH2 with a radiolabeled phosphopeptide, precipitating the complex with glutathione-Sepharose, washing the beads, and then counting the beads to determine bound radioactivity [Isakov et al., *J. Exp. Med.*, 181, 375-380 (1995); Piccione et al., *Biochemistry*, 32, 3197-3202 (1993); Huyer et al., *Biochemistry*, 34, 1040-1049 (1995)]. There are several disadvantages to this procedure, particularly when applied to high-throughput screening for agonists, antagonists, or inhibitors as new leads for drug development. First, the radiolabeling of the peptide is carried out either enzymatically with a kinase and [^{32}P]ATP or chemically with [^{125}I]Bolton-Hunter reagent. In both cases, the isotopes are short-lived and thus require frequent preparation of material. In the case of enzymatic phosphorylation, the appropriate kinase must also be available in sufficient quantity to generate enough material for screening purposes. Second, the protocol requires separation of bound complex from free phosphopeptide by washing of the glutathione-Sepharose beads. This is a nonequilibrium procedure that risks dissociation of the bound ligand, particularly when off-rates are fast. Thus, there is the possibility of misleading results. Finally, due to the number of manipulations and centrifugations involved, the protocol is very tedious to conduct manually and is not readily adaptable to robotic automation to increase throughput.

Two additional methods for measuring the interaction of proteins and ligands that have been applied to SH2 domains are biospecific interaction analysis using surface plasmon resonance and

isothermal titration calorimetry (Felder et al., *Mol. Cell. Biol.*, 13, 1449-1455 (1993); Panayotou et al., *Mol. Cell. Biol.*, 13, 3567-3576 (1993); Payne et al., *Proc. Natl. Acad. Sci. U.S.A.*, 90, 4902-4906 (1993); Morelock et al., *J. Med. Chem.* 38, 1309-18 (1995); Ladbury et al., *Proc. Natl. Acad. Sci. U.S.A.*, 92, 3199-3203 (1995); Lemmon et al., *Biochemistry*, 33, 5070-5076 (1994)). These techniques do not require a particular fusion partner for the SH2 domain, but do require sophisticated instrumentation that is not amenable to high throughput screening.

SUMMARY OF THE INVENTION

The instant invention covers a method of screening for compounds capable of binding to a fusion protein which comprises combining a test compound, a tagged ligand, a fusion protein (target protein, peptide linker and FK506-binding protein), a radiolabeled ligand, and coated scintillation proximity assay (SPA) beads, and then measuring the scintillation counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay in the absence of the test compound, so as to determine the effect the test compound has on the binding of the tagged ligand. This invention provides an immediate means of making use of SPA technology for the functional assay of ligand binding to a single or multiple signal transduction domain(s), for example a phosphopeptide binding to an SH2 domain. The present invention does not require specialized radiochemical synthesis and is readily adaptable to robotic automation for high capacity screening for agonists, antagonists, and/or inhibitors.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1.

A.) Binding of the streptavidin SPA bead, biotinylated ligand and the fusion protein (SH2:FKBP), which emits a detectable signal; and

B.) Binding of the test compound and the fusion protein (SH2:FKBP), which results in no signal detection .

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of screening for compounds which preferentially bind to a target protein.

An embodiment of this invention is a method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

a) mixing a test compound, a tagged ligand, the fusion protein, a radiolabeled ligand and coated scintillation proximity assay (SPA) beads;

b) incubating the mixture for between about 1 hour and about 24 hours;

c) measuring the SPA bead-bound counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound using scintillation counting; and

d) determining the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.

The term "fusion protein" refers to a "target protein" fused to an "FK506-binding protein" (FKBP), the two proteins being separated by a "peptide linker".

A "peptide linker" may consist of a sequence containing from about 1 to about 20 amino acids, which may or may not include the sequence for a protease cleavage site. An example of a peptide linker which is a protease cleavage site is represented by the amino acid sequence GLPRGS.

The term "target protein" refers to any protein that has a defined ligand. Included within this definition of target protein are single and multiple signal transduction domains, such as, but not limited to, Src homology 1 (SH1), Src homology 2 (SH2), Src homology 3 (SH3), and pleckstrin homology (PH) domains [Hanks & Hunter, *FASEB J.*, 9, 576-596 (1995); Bolen, *Curr. Opin. Immunol.*, 7, 306-311 (1995); Kuriyan & Cowburn, *Curr. Opin. Struct. Biol.*, 3, 828-837 (1993); Cohen et al., *Cell*, 80, 237-248 (1995)]. The term "SH1 domain" refers to a family of homologous protein domains that bind ATP and catalyze tyrosine phosphorylation of peptide and protein substrates. The term "SH2 domain" refers to a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. The term "SH3 domain" refers to a family of homologous protein domains that share the common property of recognizing polyproline type II helices. The term "PH domain" refers to a family of homologous protein domains that mediate both protein-protein and protein-lipid interactions. Examples of SH2 domains which may be utilized in the method of the invention include, but are not limited to, the single and tandem SH2 domains present in the tyrosine kinases ZAP, SYK and LCK. The DNA sequences were obtained from GenBank, National Center for Biotechnology Information, National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894. The Accession Numbers for the sequences are: human ZAP (L05148); human SYK (L28824) and human LCK (X13529).

The term "tagged ligand" refers to a biotinylated or epitope tagged ligand for the target protein.

The term "radiolabeled ligand" refers to a [^{3}H]- or [^{125}I]-labeled ligand which binds to the FKBP. An example of a radiolabeled ligand useful in the instant invention is [^{3}H]-dihydroFK506.

The term "coated scintillation proximity assay beads" (SPA beads) refers to streptavidin-coated scintillation proximity assay beads when the tagged ligand is biotinylated, and to anti-epitope antibody bound to anti-antibody-coated or protein A-coated scintillation proximity assay beads when the tagged ligand is epitope-tagged.

The term "control assay" refers to the assay when performed in the presence of the tagged ligand, the fusion protein, the radiolabeled ligand and the coated scintillation proximity assay beads, but in the absence of the test compound.

The term FK506-binding proteins may include, but are not limited to, the below listed FKBPs and FKBP homologues, which include a citation to the references which disclose them. This list is not intended to limit the scope of the invention.

Mammalian

FKBP-12	Galat et al., *Eur. J. Biochem.*, 216:689-707 (1993).
FKBP-12.6	Wiederrecht, G. and F. Etzkorn *Perspectives in Drug Discovery and Design* , 2:57-84 (1994).
FKBP-13	Galat et al., *supra*; Wiederrecht and Etzkorn, *supra.*
FKBP-25	Galat et al., *supra*; Wiederrecht and Etzkorn, *supra.*
FKBP-38	Wiederrecht and Etzkorn, *supra.*
FKBP-51	Baughman et al., *Mol. Cell. Biol.*, 8, 4395-4402(1995) .
FKBP-52	Galat et al., *supra.*

Bacteria

Legionella pneumophilia	Galat et al., *supra.*
Legionella micadei	Galat et al., *supra.*
Chlamydia trachomatis	Galat et al., *supra.*
E. coli fkpa	Horne, S.M. and K.D. Young, *Arch. Microbiol.*, 163:357-365 (1995).
E. coli slyD	Roof et al., *J. Biol. Chem.* 269:2902-2910 (1994).
E. coli orf149	Trandinh et al., *FASEB J.* 6:3410-3420 (1992).

Neisseria meningitidis	Hacker, J. and G. Fischer, *Mol. Micro.*, 10:445-456 (1993).
Streptomyces chrysomallus	Hacker and Fischer, *supra.*

Fungal

yeast FKBP-12	Cardenas et al., *Perspectives in Drug Discovery and Design* , 2:103-126 (1994).
yeast FKBP-13	Cardenas et al., *supra.*
yeast NPR1(FPR3)	Cardenas et al., *supra.*
Neurospora	Galat et al., *supra.*

A variety of host cells may be used in this invention, which include, but are not limited to, bacteria, yeast, bluegreen algae, plant cells, insect cells and animal cells.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express genes in a variety of host cells, such as, bacteria, yeast, bluegreen algae, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector may contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Commercially available vectors suitable for FKBP fusion protein expression include, but are not limited to pBR322 (Promega), pGEX (Amersham), pT7 (USB), pET (Novagen), pIBI (IBI), pProEX-1 (Gibco/BRL), pBluescript II (Stratagene), pTZ18R and

pTZ19R (USB), pSE420 (Invitrogen), pVL1392 (Invitrogen), pBlueBac (Invitrogen), pBAcPAK (Clontech), pHIL (Invitrogen), pYES2 (Invitrogen), pCDNA (Invitrogen), pREP (Invitrogen) or the like.

The expression vector may be introduced into host cells via any one of a number of techinques including but not limited to transformation, transfection, infection, protoplast fusion, and electroporation.

E. coli containing an expression plasmid with the target gene fused to FKBP are grown and appropriately induced. The cells are then pelleted and resuspended in a suitable buffer. Although FKBP-12 lacks sequences that specifically direct it to the periplasm, FKBP fusions are primarily located there and can be released by a standard freeze/thaw treatment of the cell pellet. Following centrifugation, the resulting supernatant contains >80% pure FKBP fusion, which if desired can be purified further by conventional methods. Alternatively, the assay is not dependent on pure protein and the initial periplasmic preparation may be used directly. A thrombin site located between FKBP and the target protein can be used as a means to cleave FKBP from the fusion; such cleaved material may be a suitable negative control for subsequent assays.

A fusion protein which contains a single or multiple SH2 domain(s) may be purified by preparing an affinity matrix consisting of biotinylated phosphopeptide coupled to avidin or streptavidin immobilized on a solid support. A freeze/thaw extract is prepared from the cells which express the fusion protein and is loaded onto the affinity matrix. The desired fusion protein is then specifically eluted with phenyl phosphate.

To assay the formation of a complex between a target protein and its ligand, the tagged ligand is mixed with the FKBP fusion protein in a suitable buffer in the presence of the radiolabeled ligand in the well of a white microplate. After a suitable incubation period to allow complex formation to occur, coated SPA beads are added to capture the tagged ligand and any bound fusion protein. The plate is sealed, incubated for a sufficient period to allow the capture to go to

completion, then counted in a multiwell scintillation counter. Screening for agonists/antagonists/inhibitors is carried out by performing the initial incubation prior to the capture step with SPA beads in the presence of a test compound(s) to determine whether they have an effect upon the binding of the tagged ligand to the fusion protein. This principle is illustrated by Figure 1.

The present invention can be understood further by the following examples, which do not constitute a limitation of the invention.

EXAMPLE 1

Process for Preparing the FKBP fusion cloning vector

General techniques for modifying and expressing genes in various host cells can be found in Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. Current Protocols in Molecular Biology (John Wiley & Sons, New York, New York, 1989). Sequences for a 3'- altered FKBP fragment that contained a glycine codon (GGT) in place of the stop (TGA) codon followed by a sequence encoding a thrombin site (Leu-Val-Pro-Arg) and *Bam*HI restriction site (GAATTC) were amplified using the polymerase chain reaction (PCR). The PCR reaction contained the following primers:5'-GATCGCCATGGGAGTGCAGGTGGAAACCATCTCCCCA-3' and 5'-TACGAATTCTGGCGTGGATCCACGCGGAACCAGACCTTCCAGT TTTAG-3' and a plasmid containing human FKBP-12 as the template. The resulting 367 base pair amplification product was ligated into the vector pCRII (Invitrogen) and the ligation mixture transformed into competent *Escherichia coli* cells. Clones containing an insert were identified using PCR with flanking vector primers. Dideoxy DNA sequencing confirmed the nucleotide sequence of one positive isolate. The altered 338 base pair FKBP fragment was excised from the pCRII plasmid using *Nco*I and *BamH*I and ligated into *Nco*I and*Bam*HI digested pET9d (Novagen) plasmid. Competent *E. coli* were transformed with the ligation mixture, and colonies containing the insert were identified using PCR with primers encoding for flanking vector sequences. The FKBP fusion cloning vector is called pET9dFKBPt.

EXAMPLE 2

Process for Preparing the FK-ZAP fusion expression vector

A DNA fragment encoding for the tandem SH2 domains of ZAP-70 was prepared by PCR to contain a *Bam*HI site at the 5'-end such that the reading frame was conserved with that of FKBP in the fusion vector. At the 3'-end, the fragment also incorporated a stop codon followed by a *Bam*HI site. The PCR reaction contained Molt-4 cDNA (Clontech) and the following primers: 5'-ATTAGGATCCATGCCAGATCCTGCAGCTCACCTGCCCT-3' and 5'-ATATGGATCCTTACCAGAGGCGTTGCT-3'. The fragment was cloned into a suitable vector, sequenced, digested with *Bam*HI, and the insert containing the SH2 domains ligated to *Bam*HI treated pET9dFKBPt, and transformed into *E. coli*. Clones containing inserts in the correct orientation were identified by PCR or restriction enzyme analysis. Plasmid DNA was prepared and used to transform BL21(DE3) cells.

EXAMPLE 3

Process for Preparing the FK-SYK fusion expression vector

The expression vector for the tandem SH2 domains of Syk fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Raji cell cDNA (Clontech) and the following primers: 5'-CAATAGGATCCATGGCCAGCAGCGGCATGGCTGA-3' and 5'-GACCTAGGATCCCTAATTAACATTTCCCTGTGTGCCGAT-3' .

EXAMPLE 4

Process for Preparing the FK-LCK fusion expression vector

The expression vector for the SH2 domain of Lck fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Molt-4 cDNA (Clontech) and the following primers:

5'-ATATGGATCCATGGCGAACAGCCTGGAGCCCGAACCCT-3'
and
5'-ATTAGGATCCTTAGGTCTGGCAGGGGCGGCTCAACCGTGT GCA-3'.

EXAMPLE 5

FK-ZAP

Step A: Process for Expression of FK-ZAP

E. coli BL21(DE3) cells containing the pET9dFKBPt/ ZapSH2 plasmid were grown in Luria-Bertani (LB) media containing 50 microgram/ml kanamycin at 37 degrees C until the optical density measured at 600 nm was 0.5-1.0. Expression of the FK-ZAP fusion protein was induced with 0.1 mM isopropyl beta-thiogalactopyranoside and the cells were grown for another 3-5 hr at 30 degrees C. They were pelleted at 4400 x g for 10 min at 4 degrees C and resuspended in 2% of the original culture volume with 100 mM tris pH 8.0 containing 1 microgram/ml each aprotinin, pepstatin, leupeptin, and bestatin. The resuspended pellet was frozen at -20 degrees C until further purification.

Step B: Process for Purification of FK-ZAP

The affinity matrix for purification of FK-ZAP was prepared by combining agarose-immobilized avidin with excess biotinylated phosphopeptide derived from the ζ1 ITAM sequence of the human T-cell receptor, biotinyl-GSNQLpYNELNLGRREEpYDVLDK, and washing out unbound peptide. Frozen cells containing FK-ZAP were thawed in warm water, refrozen on dry ice for about 25 min., then thawed again. After the addition of 0.1% octyl glucoside, 1 mM dithiothreitol (DTT) and 500 mM NaCl, the extract was centrifuged at 35,000 x g for approximately 30 minutes. The supernatant was loaded onto the phosphopeptide affinity column, at about 4° and washed with phosphate buffered saline containing 1 mM DTT and 0.1% octyl glucoside. FK-ZAP was eluted with 200 mM phenyl phosphate in the same buffer at about 37°. The protein pool was concentrated and the phenyl phosphate

removed on a desalting column. The purified FK-ZAP was stored at about -30° in 10 mM HEPES/150 mM NaCl/1 mM DTT/0.1 mM EDTA/10% glycerol.

EXAMPLE 6

FK-SYK

E. coli BL21(DE3) cells containing the pET9dFKBPt/SykSH2 plasmid were grown, induced, and harvested as described in Example 5. FK-SYK was purified using the same affinity matrix and methodology described in Example 5.

EXAMPLE 7

FK-LCK

E. coli BL21(DE3) cells containing the pET9dFKBPt/LckSH2 plasmid were grown, induced, and harvested as described in Example 5. The affinity matrix for purification of FK-LCK was prepared by combining agarose-immobilized avidin with excess biotinyl-EPQpYEEIPIYL, and washing out unbound peptide. The remaining methodology for purification was the same as Example 5.

EXAMPLE 8

Method of Screening for Antagonists of FK-ZAP

Assays were conducted at ambient temperature in a buffer consisting of 25 mM HEPES, 10 mM DTT, 0.01% TWEEN-20, pH 7.0. 10 µl of a DMSO solution of test compound(s) and 120 µl of biotinyl-phosphopeptide stock solution were dispensed into the wells of a 96-well Packard Optiplate. Next, 20 µl of a mixture of FK-ZAP protein and ^{3}H-dihydroFK506 were added to each test well. Finally, 50 µl of a 4 mg/ml suspension of SPA beads were dispensed to each well. Final concentrations of the assay components were:

25 nM biotinyl-GSNQLpYNELNLGRREEpYDVLDK
25 nM FK-ZAP fusion protein
10 nM ^{3}H-dihydroFK506 (DuPont NEN)
1.0 mg/ml streptavidin-SPA beads (Amersham)
5% DMSO

The plate was sealed and incubated between 1 and 8 hours. Bead-bound radioactivity was then measured in a Packard Topcount microplate scintillation counter.

EXAMPLE 9

Method of Screening for Antagonists of FK-SYK

The assays were conducted as set forth in Example 8, except that FK-SYK replaced FK-ZAP.

EXAMPLE 10

Method of Screening for Antagonists of FK-LCK

The assays were conducted as set forth in Example 8, except that FK-LCK replaced FK-ZAP and the tagged ligand was 25 nM biotinyl-EPQpYEEIPIYL.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1137 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CAGACCTGCG TGGTGCACTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC 120

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GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240

TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300

GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGCC AGATCCTGCA 360

GCTCACCTGC CCTTCTTCTA CGGCAGCATC TCGCGTGCCG AGGCCGAGGA GCACCTGAAG 420

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TATGTGCTGT CGCTCGTGCA CGATGTGCGC TTCCACCACT TTCCCATCGA GCGCCAGCTC 540

AACGGCACCT ACGCCATTGC CGGCGGCAAA GCGCACTGTG GACCGGCAGA GCTCTGCGAG 600

TTCTACTCGC GCGACCCCGA CGGGCTGCCC TGCAACCTGC GCAAGCCGTG CAACCGGCCG 660

TCGGGCCTCG AGCCGCAGCC GGGGGTCTTC GACTGCCTGC GAGACGCCAT GGTGCGTGAC 720

TACGTGCGCC AGACGTGGAA GCTGGAGGGC GAGGCCCTGG AGCAGGCCAT CATCAGCCAG 780

GCCCCGCAGG TGGAGAAGCT CATTGCTACG ACGGCCCACG AGCGGATGCC CTGGTACCAC 840

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AAGTTCCTGC TGAGGCCGCG GAAGGAGCAG GGCACATACG CCCTGTCCCT CATCTATGGG 960

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1155 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180

GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240

TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300

GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC CAGCAGCGGC 360

ATGGCTGACA GCGCCAACCA CCTGCCCTTC TTTTTCGGCA ACATCACCCG GGAGGAGGCA 420

GAAGATTACC TGGTCCAGGG GGGCATGAGT GATGGGCTTT ATTTGCTGCG CCAGAGCCGC 480

AACTACCTGG GTGGCTTCGC CCTGTCCGTG GCCCACGGGA GGAAGGCACA CCACTACACC 540

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GCCGACCTCT GCCACTACCA CTCCCAGGAG TCTGATGGCC TGGTCTGCCT CCTCAAGAAG 660

CCCTTCAACC GGCCCCAAGG GGTGCAGCCC AAGACTGGGC CCTTTGAGGA TTTGAAGGAA 720

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GGAAATGTTA ATTAG 1155

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 675 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC 60

CAGACCTGCG TGGTGCACTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC 120

CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180

GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240

TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300

GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC GAACAGCCTG 360

GAGCCCGAAC CCTGGTTCTT CAAGAACCTG AGCCGCAAGG ACGCGGAGCG GCAGCTCCTG 420

GCGCCCGGGA ACACTCACGG CTCCTTCCTC ATCCGGGAGA GCGAGAGCAC CGCGGGATCG 480

TTTTCACTGT CGGTCCGGGA CTTCGACCAG AACCAGGGAG AGGTGGTGAA ACATTACAAG 540

ATCCGTAATC TGGACAACGG TGGCTTCTAC ATCTCCCCTC GAATCACTTT TCCCGGCCTG 600

CATGAACTGG TCCGCCATTA CACCAATGCT TCAGATGGGC TGTGCACACG GTTGAGCCGC 660

CCCTGCCAGA CCTAA 675

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 378 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
1 5 10 15

Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
20 25 30

Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
35 40 45

Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
50 55 60

Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
65 70 75 80

Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
85 90 95

Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
100 105 110

Arg Gly Ser Met Pro Asp Pro Ala Ala His Leu Pro Phe Phe Tyr Gly
115 120 125

Ser Ile Ser Arg Ala Glu Ala Glu Glu His Leu Lys Leu Ala Gly Met
130 135 140

Ala Asp Gly Leu Phe Leu Leu Arg Gln Cys Leu Arg Ser Leu Gly Gly
145 150 155 160

Tyr Val Leu Ser Leu Val His Asp Val Arg Phe His His Phe Pro Ile
165 170 175

Glu Arg Gln Leu Asn Gly Thr Tyr Ala Ile Ala Gly Gly Lys Ala His
180 185 190

Cys Gly Pro Ala Glu Leu Cys Glu Phe Tyr Ser Arg Asp Pro Asp Gly
195 200 205

Leu Pro Cys Asn Leu Arg Lys Pro Cys Asn Arg Pro Ser Gly Leu Glu
210 215 220

Pro Gln Pro Gly Val Phe Asp Cys Leu Arg Asp Ala Met Val Arg Asp
225 230 235 240

Tyr Val Arg Gln Thr Trp Lys Leu Glu Gly Glu Ala Leu Glu Gln Ala
245 250 255

Ile Ile Ser Gln Ala Pro Gln Val Glu Lys Leu Ile Ala Thr Thr Ala
260 265 270

His Glu Arg Met Pro Trp Tyr His Ser Ser Leu Thr Arg Glu Glu Ala
275 280 285

Glu Arg Lys Leu Tyr Ser Gly Ala Gln Thr Asp Gly Lys Phe Leu Leu
290 295 300

Arg Pro Arg Lys Glu Gln Gly Thr Tyr Ala Leu Ser Leu Ile Tyr Gly
305 310 315 320

Lys Thr Val Tyr His Tyr Leu Ile Ser Gln Asp Lys Ala Gly Lys Tyr
325 330 335

Cys Ile Pro Glu Gly Thr Lys Phe Asp Thr Leu Trp Gln Leu Val Glu
340 345 350

Tyr Leu Lys Leu Lys Ala Asp Gly Leu Ile Tyr Cys Leu Lys Glu Ala
355 360 365

Cys Pro Asn Ser Ser Ala Ser Asn Ala Ser
370 375

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
1 5 10 15

Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
20 25 30

Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
35 40 45

Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
50 55 60

Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
65 70 75 80

Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
85 90 95

Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
100 105 110

Arg Gly Ser Met Ala Ser Ser Gly Met Ala Asp Ser Ala Asn His Leu
115 120 125

Pro Phe Phe Phe Gly Asn Ile Thr Arg Glu Glu Ala Glu Asp Tyr Leu
130 135 140

Val Gln Gly Gly Met Ser Asp Gly Leu Tyr Leu Leu Arg Gln Ser Arg
145 150 155 160

Asn Tyr Leu Gly Gly Phe Ala Leu Ser Val Ala His Gly Arg Lys Ala
165 170 175

His His Tyr Thr Ile Glu Arg Glu Leu Asn Gly Thr Tyr Ala Ile Ala
180 185 190

Gly Gly Arg Thr His Ala Ser Pro Ala Asp Leu Cys His Tyr His Ser
195 200 205

Gln Glu Ser Asp Gly Leu Val Cys Leu Leu Lys Lys Pro Phe Asn Arg
210 215 220

Pro Gln Gly Val Gln Pro Lys Thr Gly Pro Phe Glu Asp Leu Lys Glu
225 230 235 240

Asn Leu Ile Arg Glu Tyr Val Lys Gln Thr Trp Asn Leu Gln Gly Gln
245 250 255

Ala Leu Glu Gln Ala Ile Ile Ser Gln Lys Pro Gln Leu Glu Lys Leu
260 265 270

Ile Ala Thr Thr Ala His Glu Lys Met Pro Trp Phe His Gly Lys Ile
275 280 285

Ser Arg Glu Glu Ser Glu Gln Ile Val Leu Ile Gly Ser Lys Thr Asn
290 295 300

Gly Lys Phe Leu Ile Arg Ala Arg Asp Asn Asn Gly Ser Tyr Ala Leu
305 310 315 320

Cys Leu Leu His Glu Gly Lys Val Leu His Tyr Arg Ile Asp Lys Asp
325 330 335

Lys Thr Gly Lys Leu Ser Ile Pro Glu Gly Lys Lys Phe Asp Thr Leu
340 345 350

Trp Gln Leu Val Glu His Tyr Ser Tyr Lys Ala Asp Gly Leu Leu Arg
355 360 365

Val Leu Thr Val Pro Cys Gln Lys Ile Gly Thr Gln Gly Asn Val Asn
370 375 380

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 224 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
1 5 10 15

Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
20 25 30

Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
35 40 45

Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
50 55 60

Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
65 70 75 80

Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
85 90 95

Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
100 105 110

Arg Gly Ser Met Ala Asn Ser Leu Glu Pro Glu Pro Trp Phe Phe Lys
115 120 125

Asn Leu Ser Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro Gly Asn
130 135 140

Thr His Gly Ser Phe Leu Ile Arg Glu Ser Glu Ser Thr Ala Gly Ser
145 150 155 160

Phe Ser Leu Ser Val Arg Asp Phe Asp Gln Asn Gln Gly Glu Val Val
165 170 175

Lys His Tyr Lys Ile Arg Asn Leu Asp Asn Gly Gly Phe Tyr Ile Ser
180 185 190

Pro Arg Ile Thr Phe Pro Gly Leu His Glu Leu Val Arg His Tyr Thr
195 200 205

Asn Ala Ser Asp Gly Leu Cys Thr Arg Leu Ser Arg Pro Cys Gln Thr
210 215 220

WHAT IS CLAIMED IS:

1. A method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

a) mixing a test compound, a tagged ligand, the fusion protein, a radiolabeled ligand and coated scintillation proximity assay (SPA) beads;

b) incubating the mixture from between about 1 hour to about 24 hours;

c) measuring the SPA bead-bound counts attributable to the binding of the tagged ligand to the fusion protein in the presence of the test compound using scintillation counting; and

d) determining the binding of the tagged ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.

2. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 1, wherein the tagged ligand is a biotinylated ligand or epitope-tagged ligand.

3. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 2, wherein scintillation proximity assay beads are streptavidin-coated or anti-antibody or protein A-coated.

4. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 3, wherein the radiolabeled ligand consists of [^{3}H]- or [^{125}I]-labeled FK506 analog.

5. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 4, wherein the fusion protein comprises an FK506-binding protein linked through a peptide linker to a target protein.

6. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 5, wherein the target protein comprises a single or multiple signal transduction domain.

7. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 6, wherein the single or multiple signal transduction domain is selected from the group consisting of: SH1, SH2, SH3 and PH domains.

8. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 7, wherein the target protein is a single or multiple SH2 domain.

9. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 8, wherein the radiolabeled ligand is [^{3}H]-dihydroFK506.

10. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 9, wherein the FK506-binding protein is a 12kDA human FK506-binding protein.

11. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 10, wherein the target protein is a single or multiple SH2 domain selected from the group consisting of: ZAP:SH2, SYK:SH2 and LCK:SH2.

12. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, ZAP:SH2.

13. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, SYK:SH2.

14. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the target protein is the SH2 domain, LCK:SH2.

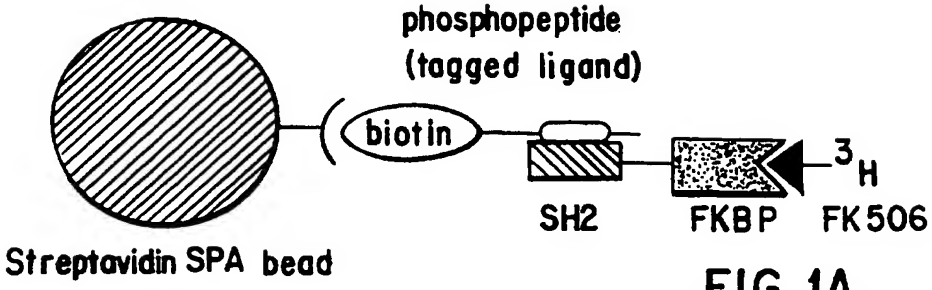
phosphopeptide
(tagged ligand)
biotin
^3H
SH2
FKBP
FK506
Signal
detected
Streptavidin SPA bead

FIG. 1A

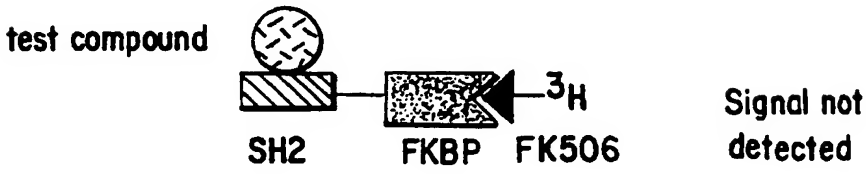
test compound
^3H
SH2
FKBP
FK506
Signal not
detected

FIG. 1B

INTERNATIONAL SEARCH REPORT

Inte. ional application No.
PCT/US96/14563

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53, 33/534
US CL : 435/7.5; 436/529
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.5; 436/529

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS
search terms: scintillation proximity assay#, spa, fusion protein#, sh2 domain#, transduction domain#, target protein#, biotin?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Sonatore et al. The Utility of FK506-Binding Protein as a Fusion Partner in Scintillation Proximity Assays: Application to SH2 Domains. Anal. Biochem. 1996, Vol. 240, pages 289-297.	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 DECEMBER 1996	Date of mailing of the international search report 10 JAN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ROSEMARY ASHTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int. tional application No.
PCT/US96/14563

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Lerner et al. Scintillation Proximity Assay for Human DNA Topoisomerase I using Recombinant Biotiny Fusion Protein Produced in Baculovirus-Infected Insect Cells. Anal. Biochem. 1996, Vol. 240, pages 289-297.	
Y	Skinner et al. Direct Measurement of the Binding of RAS to Neurobromin using a Scintillation Proximity Assay. Anal. Biochem. 1994, Vol. 223, pages 259-265.	1,3